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Cytotoxic factor-autoantibodies: possible role in the pathogenesis of dengue haemorrhagic fever

U.C. Chaturvedi [a,*], E.A. Elbishbishi [a], R. Agarwal [b], A.S. Mustafa [a]

[a] *Department of Microbiology, Faculty of Medicine, Kuwait University, P.O. Box 24923, 13110 Safat, Kuwait*
[b] *Department of Microbiology, Sanjay Gandhi P.G.I.M.S., 226014 Lucknow, India*

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Abstract

During dengue virus infection a unique cytokine, cytotoxic factor (hCF), is produced that is pathogenesis-related and plays a key role in the development of dengue haemorrhagic fever (DHF). However, what regulates the adverse effects of hCF is not known. We have previously shown that anti-hCF antibodies raised in mice, neutralise the pathogenic effects of hCF. In this study we have investigated the presence and levels of hCF-autoantibodies in sera of patients with various severity of dengue illness ($n = 136$) and normal healthy controls ($n = 50$). The highest levels of hCF-autoantibodies (mean ± S.D. = 36 ± 20 U ml^{-1}) were seen in patients with mild illness, the dengue fever (DF), and 48 out of 50 (96%) of the sera were positive. On the other hand the hCF-autoantibody levels declined sharply with the development of DHF and the levels were lowest in patients with DHF grade IV (mean ± S.D. = 5 ± 2 U ml^{-1}; $P = < 0.001$ as compared to DF). Only one of the 13 DHF grade IV patients had an antibody level above the 'cut-off' value (mean plus 3 S.D. of the control sera). The analysis of data with respect to different days of illness further showed that the highest levels of hCF-autoantibodies were present in DF patients at > 9 days of illness. Moreover, the DF patients at all time points, i.e. 1–4, 5–8 and > 9 days of illness had significantly higher levels of hCF-autoantibodies ($P < 0.001$) than patients with DHF grade I, II, III and IV. In addition DHF grade I and grade II patients had significantly more positive specimens than DHF grade III and grade IV patients at all time points. These results suggest that elevated levels of hCF-autoantibodies protect the patients against the development of severe forms of DHF and, therefore, it may be useful as a prognostic indicator.

Keywords: Cytokine; Cytotoxic factor; Dengue; Dengue haemorrhagic fever; Pathogenesis; Autoantibody

1. Introduction

Dengue virus infection produces a mild self-limiting acute febrile illness, dengue fever (DF), and a life-threatening severe illness, dengue haemorrhagic fever (DHF) with minor or major bleeding from different sites. The main features of DHF are increased capillary permeability leading to extensive plasma leakage resulting in profound shock, increased haematocrit, thrombocytopoenia and altered number and functions of leucocytes. DHF has been classified into four grades on the basis of clinical presentation and laboratory findings; the mildest is grade I and the most severe is grade IV [1–3]. Despite extensive studies, the pathogenesis of DHF is still not fully understood. We have observed that the most significant findings in patients with severe DHF are high levels of the cytotoxic factor (hCF) in the sera and peripheral blood leucocytes [4] and a shift from the Th1-type response to Th2-type response [5].

During dengue virus infection the CD4$^+$ T cells produce a unique cytokine, cytotoxic factor (CF), in mice (mCF) and its homologue in man (hCF). The amino-terminal sequence of mCF has no homology with any known proteins or cytokines. mCF and hCF appear to be pathogenesis-related proteins, capable of reproducing DHF-like pathological lesions in mice, such as increased capillary permeability, cerebral oedema and blood leukocyte changes [3,6–8]. During an extensive epidemic of DHF in Northern India during 1996, the presence of hCF was shown in 90% of the 333 patients with peak amounts in the most severe patients with DHF grade IV(9). Further, ex vivo culture of peripheral blood mononuclear cells of such patients showed the production of hCF by CD4$^+$ T cells [4,10]. The production of mCF/hCF precedes the clinical illness in mice and man [4,7–9].

We have recently proposed a mechanism to explain the

* Corresponding author. Present address: CSIR Emeritus Scientist, Industrial Toxicology Research Centre, M.G. Road, P.O. Box No. 80, Lucknow-226001, India. Tel.: +91-(522)-372975/372770; Fax: +91-(522)-228227.

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pathogenesis of DHF in which hCF plays a key role [11]. Dengue virus replicates in macrophages and induces quickly the $CD4^+$ T cells to produce hCF. hCF induces macrophages to produce free radicals, nitrite, reactive oxygen and peroxynitrite [12,13]. The free radicals, besides killing the target cells by apoptosis, also directly upregulate production of proinflammatory cytokines interleukin (IL)-1α, tumour necrosis factor (TNF)-α, IL-8, and hydrogen peroxide in macrophages. The change in relative levels of IL-12 and TGF-α shifts a Th1-dominant response to a Th2-biased response resulting in an exacerbation of dengue disease and death of patients. The vascular permeability is increased due to the combined effect of histamine, free radicals, proinflammatory cytokines and the products of the complement pathway, etc. Thus, the key player appears to be hCF, but what regulates its activity is not known [11].

Anti-cytokine antibodies can neutralise the adverse effects of cytokines, including hCF [7], while the non-neutralising type of antibodies may contribute to better targeting of the cytokines to the appropriate cells [14,15]. Thus, autoantibodies may be a potent regulator of cytokine functions. The present study was, therefore, undertaken to investigate the sera of the patients with DF/DHF for the presence of hCF-autoantibodies.

2. Materials and methods

2.1. Patients

An extensive epidemic of DHF occurred in Northern India during August–November 1996. During this epidemic serum samples were collected from the patients suffering from typical dengue-like illness admitted to the Gandhi Memorial and Associated Hospitals, Lucknow and the Pediatrics Department of the All India Institute of Medical Sciences, New Delhi. Diagnosis of dengue virus infection was established either by virus isolation or by detection of virus-specific IgM in the sera [5]. Sera were available from a total of 136 patients for the present study. For controls, 50 age-matched normal healthy individuals, without history of any febrile or other illnesses in the previous 3 months, were included. Among the patients, 50 were classified as DF, 10 as DHF grade I, 50 as grade II, 13 as grade III and 13 as grade IV, according to the criteria of the World Health Organisation [2]. Sera collected from the patients and controls were divided in aliquots and quickly frozen and stored at −60°C. For the present study, sera were transported to Kuwait on dry ice and stored at −70°C until tested.

2.2. Enzyme-linked immunosorbent assay (ELISA)

The ELISA for the detection of hCF and its antibody has been standardised and described earlier [16]. Polystyrene flat-bottom 96-well plates (Polysorb F96, Nunc, Glostrup, Denmark) were used. The hCF used in the test was purified from the sera collected from dengue patients using ion-exchange chromatography followed by high performance liquid chromatography (HPLC) as described [7]. The antibody against HPLC-purified hCF were prepared in mice [7]. The anti-hCF antibody (hCFAS) neutralises the cytotoxic activity of hCF in vitro and inhibits hCF-induced increase in capillary permeability in mice and reacted specifically with hCF in the Western blot tests and ELISA. The controls included were purified hCF as positive control and heterologous proteins as negative controls [7,8].

The sandwich ELISA was optimised by a Checkerboard titration to find the minimum amount of hCF (on the solid phase) that reacted with a minimum amount of hCFAS. The antibody-binding curve indicated that a concentration of 300 ng ml^{-1} hCF was optimum to coat the solid phase. This concentration of hCF coating gave a hCFAS binding curve with the 50% maximum binding point at the dilution of 1:500 of hCFAS. In the test system the recovery rates were within 10% and the variations in the intra-assay and inter-assay were less than 10%. The established ELISA was reproducible and the specificity, sensitivity and accuracy were 92.5, 90.9 and 91%, respectively [16]. To control day-to-day variations in ELISA, the optical density (OD) values of the standard sample tested undiluted and at 1/10, 1/100 and 1/1000 dilutions were converted into arbitrary units and used to construct a standard curve. Each sample was diluted 10-fold and the test was setup in triplicate. The OD values obtained were converted into arbitrary units ml^{-1} (U ml^{-1}) by comparison with the standard curve. The data has been presented as U ml^{-1} of hCFAS and analysed statistically using Student's *t*-test. A *P*-value of less than 0.05 was considered significant.

3. Results

The hCF-autoantibody levels in patients with various grades of dengue illness have been presented as mean values of the arbitrary U ml^{-1} in Fig. 1. In patients with mild illness, DF, the mean value of the hCF-autoantibodies was 36 ± 20 U ml^{-1}, the highest value being 109 U ml^{-1} in one patient. The hCF-autoantibody levels declined sharply with the onset of DHF. In patients with DHF grade I, grade II, grade III and grade IV the mean values were 14 ± 7, 10 ± 5, 6 ± 2.5 and 5 ± 2 U ml^{-1} ($P = < 0.001$, as compared to DF patients). Among the 50 normal healthy control sera the mean value for hCF-autoantibodies was 2 ± 1 U ml^{-1} (Fig. 1). To exclude the possibility of detecting non-specific antibodies in the sera of the patients and controls due to polyclonal activation of B cells, all the samples were tested in a parallel ELISA using ovalbumin as antigen on the solid phase. The OD values of all the

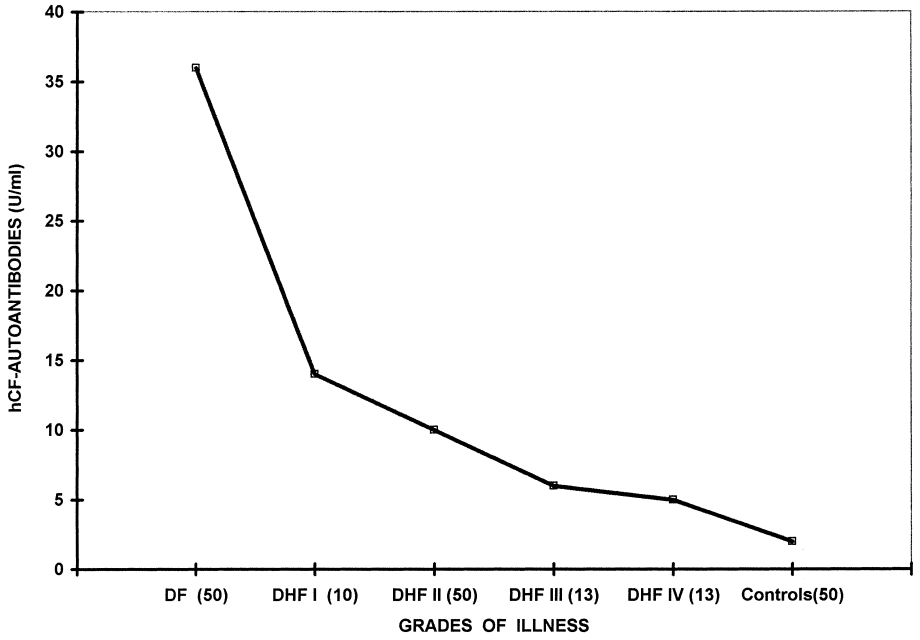

Fig. 1. Levels of hCF-autoantibodies in cases of dengue. Sera collected from the patients of various grades of the dengue illness were screened for hCF-autoantibodies concentration by sandwich ELISA. The mean value of the data have been presented as arbitrary units (U ml^{-1}). The figures in the parentheses represent the total number of patients/controls in each group.

sera in these tests were close to the OD values in the blank wells (data not shown).

The mean value of hCF-autoantibodies in the control sera plus 3 S.D. was considered as the 'cut-off' value. A patient with dengue illness was considered positive if the level of hCF-autoantibodies was more than the 'cut-off' value. The analysis of the data presented in Fig. 2 shows that 48/50 (96%) patients with DF were positive for hCF-autoantibodies, while 8/10 (80%) patients with DHF grade I, 27/50 patients (54%) with DHF grade II, 2/13 patients (15%) with DHF grade III and 1/13 patients (8%) with DHF grade IV were positive. Thus, maximum positivity was seen in DF patients and it decreased gradually from less severe to more severe forms of DHF.

The hCF-autoantibody levels in different grades of dengue disease were further analysed with respect to the

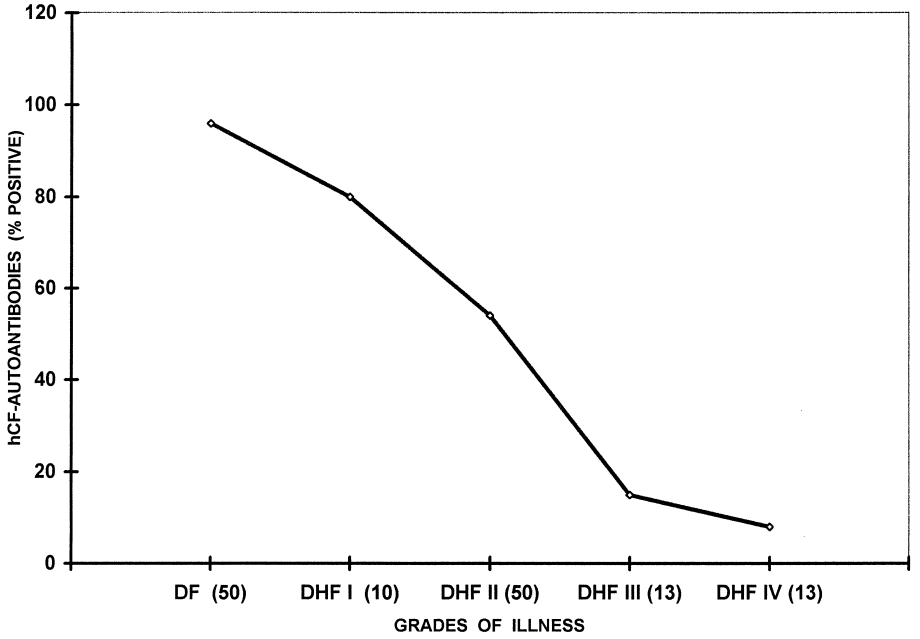

Fig. 2. Percentage of hCF-autoantibody-positive patients as a function of the stage of dengue illness. Mean values from the 50 normal healthy control sera plus 3 S.D. was taken as a 'cut-off' value to designate a serum sample from a patient as positive. The figures in the parentheses represent total number of the patients in each group.

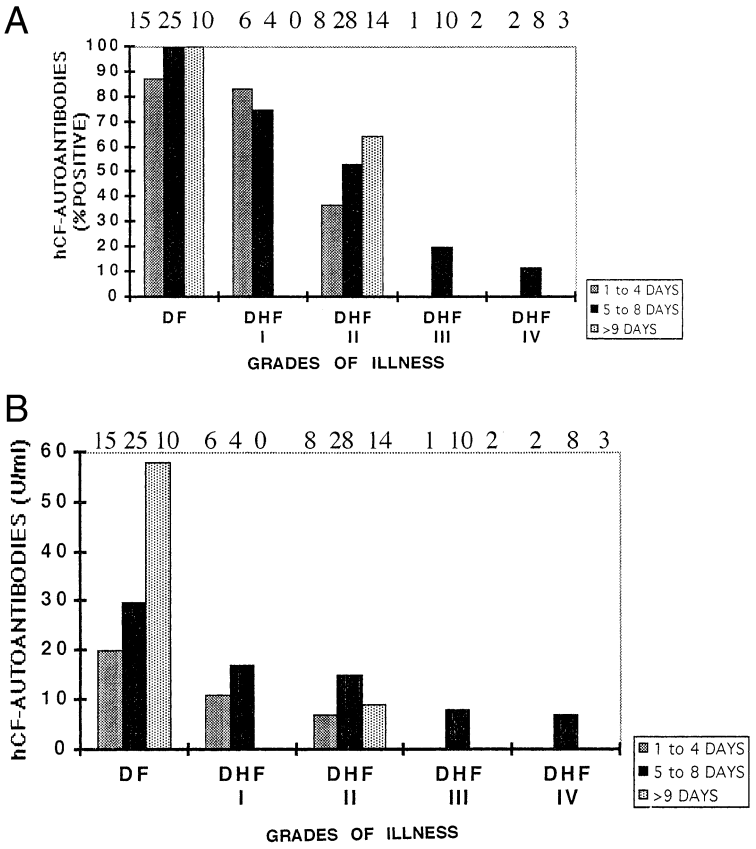

Fig. 3. hCF-autoantibodies as a function of the duration and the grades of dengue illness. (A) Percentage of hCF-autoantibodies positive patients and (B) levels of hCF-autoantibodies (U ml^{-1}) in the patients. The figures above the columns represent the total number of patients in each group.

duration of illness by dividing the patients into three groups, i.e. patients with 1–4 days, 5–8 days and >9 days of illness. The results showed that the percentage of positive samples in individual groups of patients on different days of illness were somewhat similar (Fig. 3A). However, in DF patients, the level of hCF-autoantibodies at >9 days was significantly higher ($P < 0.001$) than the levels at 1–4 and 5–8 days of illness. Moreover, DF patients had significantly higher levels of hCF-autoantibodies ($P < 0.001$) in their sera at all time points when compared with DHF patients of grades I to IV (Fig. 3B). Similarly, the patients with DHF grade I had significantly elevated levels of hCF-autoantibodies in their sera on days 1–4 and 5–8 when compared with sera from DHF grade II, grade III and grade IV (Fig. 3B).

4. Discussion

The most significant finding of the present study is the presence of hCF-autoantibodies in maximum amounts and in a maximum number of the patients with the mild dengue disease (DF). On the other hand, the levels of hCF-autoantibodies and the number of sera positive for the autoantibodies were lowest in patients with severe disease, the DHF grades III and IV. The differences in the level of autoantibodies between DF and DHF grades III and IV were highly significant ($P = < 0.001$). Thus, low levels of the hCF-autoantibodies correlated directly with the severity of the disease. Sera from normal healthy control individuals had minimal amounts of hCF-autoantibodies and their mean value plus 3 S.D. was used as 'cut-off' value. It is noteworthy that, except for dengue, hCF/mCF has been detected in no other infection. Further, the amino-terminal sequence of mCF does not match with any cytokine or known protein sequences available in the database [3]. hCF is a cytokine produced by $CD4^+$ T cells in response to dengue virus infection. It has been proposed to be involved in the pathogenesis of DHF. In general, cytokines are required for a number of normal functions in the body but may produce pathological effects when they are present in wrong places and at wrong concentrations. The presence of autoantibodies to a number of cytokines has been reported and is known to block the activity of the cytokines [17]. Natural IgG antibodies are found in the serum for IL-1α, IL-6, IL-10, interferon (IFN)-α, IFN-β and GM-CSF, (reviewed in [14,15]). Small complexes between such autoantibodies and cytokines do not activate complement if IgG4 antibodies are involved, and do not precipitate in vivo, but are active as inflammatory complexes [17].

The anti-hCF/mCF antibodies raised in mice react spe-

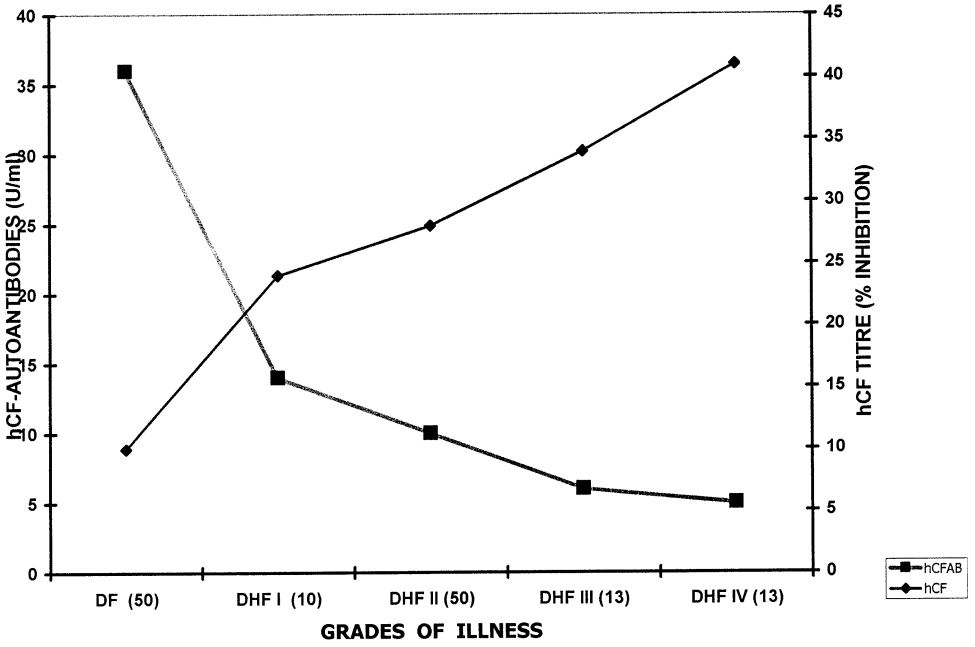

Fig. 4. Comparison of the levels of hCF-autoantibodies with those of hCF (see [9]) in the sera of the patients with dengue in relation to the grades of illness.

cifically with hCF/mCF in the Western blot tests and ELISA, neutralise the cytotoxic activity of hCF/mCF in vitro and inhibit hCF/mCF-induced increase in capillary permeability in mice and, thus, protect mice against mCF/hCF-induced DHF-like pathological lesions [6,7,18]. Furthermore, active vaccination of mice using mCF as antigen protects them against subsequent challenge with mCF. Challenge of such mice with a lethal intracerebral dose of dengue virus fails to manifest clinical symptoms of the disease [19]. Recently, the production of neutralising autoantibodies has been reported by vaccination with IL-1α [20]. Similar strategies are being successfully used in several diseases using anti-TNF-α antibody therapy [21]. The cytokine-autoantibodies specifically neutralise their corresponding cytokines in vitro but in vivo they may facilitate functions of cytokines in the body by acting as specific physiological carriers or in cytokine-protective or -stabilising functions [14,15]. Therefore, it may be argued that the hCF-autoantibodies are targeting hCF to the appropriate cells and are thus consumed and have low levels in severe disease. In another study, carried out on the same set of sera, the levels of hCF were investigated by inhibition ELISA [9]. The findings showed the lowest levels of hCF in patients with DF and the highest in those with DHF grade IV [9]. A comparison of the hCF [9] and hCF-autoantibody levels in the sera, as observed in the present study, is presented in Fig. 4. The high hCF-autoantibody levels in patients with DF are associated with the low levels of hCF. On the other hand, high hCF levels in patients with DHF grade IV are associated with low levels of hCF-autoantibodies. Thus, a reverse correlation existed between the two. These findings and the available data as presented above strongly support the view that the presence of high levels of hCF-autoantibodies, that are neutralising, may protect against development of severe DHF. This is in line with the conclusions drawn with autoantibodies against IL-1α in patients with chronic polyarthritis [22].

Another interesting finding that has emerged from the present study is that the levels of the hCF-autoantibodies may be used as an indicator of prognosis in patients with dengue disease. If the hCF-autoantibody levels are high in a patient, the chances are that he/she may recover without developing DHF. However, follow-up studies, at different time periods after the onset of clinical illness, are needed to extend these suggestions.

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